RESEARCH ARTICLE

Comparative morpho-physiological response of *in vitro* selected somaclones of wheat (*Triticum aestivum* L.) and explant donor parent to drought stress

Imran Mahmood[1]*, Abdul Razzaq[1], Abdul Ahad Qureshi[2], Abdul Qayyum[3] and M.M. Qadeer Baig[4]
[1] *Department of Agronomy, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi-46300, Pakistan.*
[2] *Department of Horticulture, PMAS-Arid Agriculture University (Sub-campus Khushab), Rawalpindi-46300, Pakistan.*
[3] *Department of Agronomy, University of Haripur, Haripur, Pakistan.*
[4] *Adaptive Research Farm Gujranwala, Pakistan.*

Abstract: Drought stress is the most prevalent environmental factor limiting wheat productivity globally and demands integration of new plant improvement approaches. Plant tissue culture, being a mutagenic process induces somaclonal variations, which can be manipulated for improving drought tolerance of wheat. An *in vitro* selection system was employed to regenerate PEG-6000 tolerant callus lines into R_1 somaclones. Comparative morpho-physiologcial responses of R_1 somaclones and explant donor parent cultivar to osmotic stress were studied at seedling and booting stages by inducing artificial drought stress along with the control of unstressed. Both R_1 somaclones and explant donor parent exhibited differential responses to drought stress at both growth stages and the selected somaclones were less affected by drought stress over their donor parent. At seedling stage, the R_1 somaclones exhibited significantly higher root length, shoot length, root to shoot length ratio and relative water contents (RWC) under PEG-6000 induced osmotic stress. Similarly, at booting stage the R_1 somaclones accumulated significantly higher proline, protein, sugar and potassium ion (K^+) contents and maintained higher membrane stability index (MSI) than the original cv. GA-2002 under drought stress conditions. The results revealed that the R_1 somaclones of wheat regenerated from PEG-6000 tolerant calli have better drought tolerance than the explant donor parent cv. GA-2002, suggesting that *in vitro* immature embryo culture can be employed to produce drought tolerant plants of wheat.

Keywords: Callus culture, osmotic stress, plant physiology, regeneration, somaclonal variations.

INTRODUCTION

Wheat is one of the leading cereal crops of the world. It provides 20 % of the world's caloric consumption, and for the 50 % of the world's poorest, 20 % of their protein consumption (Washington Grain Commission, 2015 – 2016). Drought stress adversely affects growth, development and also physiological and biochemical processes, and is a stern threat for sustainable crop production world-wide (Farooq *et al.*, 2009). Half of the world's arable land is arid or susceptible to drought. Thus, it is an imperative aim of the plant breeders to develop drought tolerant cultivars (Mahmood *et al.*, 2014) of cereals to feed the world's ever growing population.

Integration of physiological traits, genetic and genomic tools and transgenic approaches may help to improve resistance against drought in wheat. However, advances in tissue culture techniques, especially callus culture have widened the scope of physiological crop improvement and is an alternative tool for developing stress-tolerant cultivars with limited time, space and cost (Mahmood *et al.*, 2012a; 2014).

During *in vitro* culture some regenerants appeared to be no longer precise clonal copies of their parents (Prematilake, 2010; Mahmood *et al.*, 2014). The genetic

or phenotypic variations among such plants are termed as somaclonal variations and sometimes provide a very important source of genetic variations for desired traits including drought tolerance, disease resistance, improved quality, quantity and yield parameters (Matheka *et al*., 2008; Mahmood *et al*., 2012a; 2014). The natural variations for drought-tolerance among the somaclones can be exploited *in vitro* in the presence of a suitable selection pressure of osmotic agent and stress duration (Mahmood *et al*., 2012b).

The choice of a suitable selection agent with appropriate selection pressure and stress duration is a prerequisite to screen drought tolerant callus lines, and subsequently regenerate them into drought tolerant somaclones (Mahmood *et al*., 2012b; Verma *et al*., 2013). Polyethyleneglycol, mol.wt. 6000 (PEG-6000), a non-ionic, non-penetrating and nontoxic water soluble polymer of high molecular weight has long been used for this purpose. It lowers the water potential of the nutrient medium similar to soil drying without being taken up by the plants or being phytotoxic and has been used effectively to explore somaclonal variations for the improvement of crops like maize (El-Aref, 2002; Matheka *et al*., 2008), wheat (Hsissou & Bouharmont, 1994; Mahmood *et al*., 2012a; 2014), rice (Verma *et al*., 2013) and sugarcane (Taghian, 2002) against drought stress.

Drought tolerance is a complex trait and to validate whether the selected somaclones regenerated from PEG-6000 tolerant calli possess higher drought tolerance than the parents, they are to be compared with their donor parents based on various physiological drought tolerance indices (Mahmood *et al*., 2014).

Visualising the scope of somaclonal variations, the present study was planned to explore the potential of *in vitro* somaclonal variations for improving drought tolerance of wheat by employing immature embryo culture and PEG-6000 as the screening agent. The morpho-physiological responses of R_1 selected somaclones and original parents in response to simulated drought stress are discussed.

METHODOLOGY

Plant material

The seeds of wheat cultivar GA-2002 (cv. GA-2002) obtained from the National Agricultural Research Centre (NARC) were surface sterilised with 70 % ethanol for 5 min and sown in the pots.

Tissue culture procedure

The spikes were harvested 2 – 3 wks post-anthesis to collect immature embryos. The caryopses were surface sterilised for 5 min with 90 % ethanol followed by washing with three to four changes of sterilised distilled water. The seeds were again sterilised for about 30 min with 6.5 % solution of NaOCl containing a few drops of 0.1 % Tween-20, followed by thorough washing with sterile distilled water. Immature embryos were removed aseptically and cultured on MS medium (Murashige & Skoog, 1962) supplemented with 4.0 mgL^{-1} 2,4-D keeping the scutella side upward (Mahmood *et al*., 2012c). All the tissue culture media were enriched with 30.0 gL^{-1} sucrose and 6.0 gL^{-1} agar. The cultures were enriched at 25 ± 1 ºC in complete darkness for 3 wks for callus induction. The induced calli were proliferated on MS medium supplemented with 2.0 mgL^{-1} 2,4-D for another 3 wks. Six explants were cultured in each culture jar of 250 mL capacity, containing 50 mL of media. The callus induction and proliferation media were replaced with fresh media after every 12 – 14 ds.

Selection of osmotic stress tolerant calli and regeneration of drought tolerant somaclones (R_0 plants) to produce R_1 seed

In vitro selection and regeneration procedure for osmotic stress tolerant somaclones (R_1) reported by Mahmood *et al*. (2012a; 2014) for wheat was followed. The proliferated calli were divided into micro-clumps of approximately 100.0 ± 10.0 mg and were cultured on callus selection medium, which is MS medium supplemented with 2.0 gL^{-1} 2,4-D and PEG-6000 induced osmotic stress of - 0.9 MPa for 4 wks to select osmotic stress tolerant callus lines. After 4 wks embryogenic out-growths from the surviving calli were excised and proliferated on fresh callus proliferation medium (MS medium supplemented with 2.0 mgL^{-1} 2,4-D) devoid of PEG-6000 for 2 wks. The micro-clumps of selected callus pieces were again transferred onto fresh callus selection media for another selection cycle of 4 wks in order to avoid any chance of escaping non-tolerant callus lines. After 2 selection cycles, necrotic tissues were separated from calli and surviving aggregates of cells (callus) were considered osmotic stress tolerant and designated as selected drought tolerant callus lines.

The selected drought tolerant callus lines were cultured on standardised regeneration medium for wheat cv. GA-2002 (MS medium supplemented with 0.5 mgL^{-1} kinetin + 0.2 mgL^{-1} IAA + 0.5 mgL^{-1} BAP) for shoot proliferation and incubated at 26 ± 1 ºC with 8 h dark

and 16 h light photoperiod. The regenerated shoots were transferred onto half strength MS medium for root induction. The regenerated plantlets (R_0) were hardened and transferred to earthen pots under control conditions, grown to maturity and selfed to produce R_0 seeds.

Appraisal of drought tolerance of R_1 somaclones

Drought tolerance of the selected R_1 somaclones (progeny of R_0 seeds) was compared with the original parent cv. GA-2002 at seedling stage and booting stage. Accordingly, the R_0 seeds of somaclones and seeds of their parent cv. GA-2002 were surface sterilised, germinated in Petri plates for 2 – 3 ds and cultured in plastic boxes ($18 \times 14 \times 4$ cm³) filled with sand. Five seedlings were cultured in each box and 100 % field capacity was maintained in the boxes by applying distilled water. One week after transferring the seedlings into boxes, out of the total, half number of the boxes were provided with half-strength Hoagland solution containing 20 % PEG-6000 (V/W) as stressing agent, while the remaining half number was kept as control (no PEG-6000 added). The plants were then supplied with distilled water to maintain 100 % field capacity daily for 15 ds after transferring the seedlings into the boxes, and various drought adopted seedling traits like root length (cm), shoot length (cm), root to shoot length ratio (root:shoot) and relative water content (RWC) were recorded.

In the 2nd step of the study, the germinated seeds were established, uniform seedlings were selected and transferred into earthen pots of 18.5 L capacity (r = 14 cm; h = 30 cm) filled with sandy loam soil. Three pots per treatment and 4 plants per pot were maintained and were provided with equal amount of water daily to maintain 100 % field capacity. After 6 wks of seedling transfer into the pots (booting stage), drought stress was induced by withholding water completely for a specific period and sampling was done on the 2nd, 4th, 6th and 8th day of withholding water to study the physiological responses of plants. At the 9th day of withholding water, the leaves of both R_1 somaclones and parent cultivar showed severe symptoms of wilting and no further sampling was carried out. Sampling was also done at zero day of drought (control). The following physiological drought indices were studied.

Relative water content (RWC) of seedling

The plants were uprooted with intact roots and fresh weight (F_W) was immediately taken. Then the plants were submerged for 4 h in distilled water at room temperature under constant light. After soaking for 4 h turgid weight (T_W) was taken. The plants were then dried for 36 h at 70 ºC for dry weight (D_W). Plant RWC was estimated as:

$$RWC\ (\%) = \left[\frac{F_W - D_W}{T_W - D_W}\right] \times 100$$

Free proline content ($\mu mol g^{-1}$ fr. wt.)

The free proline content was estimated by the method of Bates *et al*. (1973) using toluene as blank. Leaf samples (500.0 mg) were crushed and homogenised in 3 % sulfosalicylic acid (5.0 mL) with the help of mortar and pestle. The homogenised samples were filtered (Whatman no.1 filter paper) and volume of the filtrate was raised to 10.0 mL with sulfosalicylic acid. Then, to 2.0 mL of the extract in a test tube 2.0 mL of ninhydrin reagent and 2.0 mL of glacial acetic acid (1.25 g of ninhydrin dissolved in a blend of 20.0 mL of ortho-phosphoric acid and 30.0 mL of glacial acetic acid) was added. The resultant mixture was boiled for a period of 30 min in a water bath at 100 ºC. After cooling the reaction mixture, 6.0 mL of toluene was added and mixed thoroughly. After mixing, the chromophore containing toluene was separated and absorbance of the sample was recorded against toluene blank at 520 nm by spectrophotometer (Optima, SP 3000 plus).

Total soluble sugar content ($mg g^{-1}$ dry wt.)

The total soluble sugar content was estimated from dry leaf samples by phenol sulphuric acid reagent method described by Dubois *et al*. (1951). Leaf samples (0.5 g) were extracted in phosphate buffer, 1.0 mL of extract was mixed with 0.5 mL of 5 % phenol solution and then 2.5 mL of 96 % sulphuric acid was added rapidly. The tube was gently agitated during acid addition and then allowed to stand in a water bath at 26 – 30 ºC for 20 min. The absorbance of coloured solution was measured at 490 nm by spectrophotometer (Optima, SP 3000 plus) using glucose as the standard.

Total soluble protein content ($mg g^{-1}$ dry wt.)

Total soluble protein content was determined by employing the method of Lowry *et al*. (1951). The dried leaf sample (250.0 mg) was macerated with 10.0 mL of phosphate buffer solution. The content was centrifuged at 3000 rpm for 10 min and the supernatant was collected and made up to 25.0 mL. To 1.0 mL of the supernatant, 5.0 mL of alkaline copper tartarate reagent and 0.5 mL of folin reagent were added. The colour intensity was measured at 660 nm (spectrophotometer; Optima, SP 3000 plus) and soluble protein was quantified by using bovine serum albumin as the standard.

Potassium content (mgg⁻¹ dry wt.)

Dried and ground leaf samples (1.0 g) were collected in 100 mL flasks and 25.0 mL of 1.0 N HCl was added. The flasks were stored for 24 h and filtered using Whatman no.1 filter paper. K^+ content of the filtrate was estimated by flame photometer using K^+ standards (0, 20, 40, 60, 80 and 100 ppm; Yoshida *et al*., 1972).

Chlorophyll content (mgg⁻¹ fresh wt.)

A fresh sample of leaves was ground with mortar and pestle in 80 % acetone and was kept at room temperature for 3 – 4 h in dark to avoid any photobleaching and for complete extraction of pigments. The mixture was centrifuged at 12000 rpm and absorbance of the supernatant was measured by spectrophotometer. For chlorophyll a and chlorophyll b absorbance (A) was recorded at 647 nm and 664 nm, respectively using PFP-7 flame photometer. Total chlorophyll was quantified by the method of Coombs *et al*. (1987).

Total chlorophyll = 7.93 A664 + 19.53 A647

Percentage leaf membrane stability index (MSI)

Percentage leaf membrane stability index was estimated by adopting the method used by Chandrasekar *et al*. (2000). Double distilled water (10.0 mL) was taken separately in two different sets of test tubes. Leaf samples (200.0 mg) cut into fine pieces were added to each set of test tubes. One set of test tubes was heated at 40 ºC for 30 min in a water bath and electrical conductivity of the sample was recorded (C_1). The second set of test tubes was incubated for 15 min at 100 ºC in a water bath and their electrical conductivity (EC) was recorded as above (C_2). MSI was calculated by using the following formula:

$$MSI\ \% = \left[1 - \frac{C_1}{C_2}\right] \times 100$$

Layout of experiment and data analysis

The first experiment was designed as completely randomised design (CRD) and the second experiment (pot trail) was laid out following randomised complete block design (RCBD) with factorial arrangement repeated thrice. The data were analysed using Statistix 8.1.1.0 software and treatment means were compared by least significant difference (LSD) test at α = 0.05.

RESULTS AND DISCUSSION

Water deficit is a severe environmental stress affecting plant productivity with an evident effect on plant growth and development. The morpho-physiological responses of R_1 somaclones and explant donor parent cv. GA-2002 were studied at seedling and booting stages in response to 20 % PEG-6000 and various durations of drought stress, respectively.

Osmotic stress at the seedling stage of both R_1 somaclones and explant donor parent cv. GA-2002 showed pronounced effect on root length (RL), shoot length (SL) root to shoot length ratio (root:shoot) and RWC of seedlings, with significant decrease in the studied traits compared with the control. Under controlled conditions, non-significant differences were witnessed for the studied traits. The R_1 somaclones showed significantly higher RL and SL and maintained higher RWC in response to 20 % PEG-6000 than the explant donor parent cv. GA-2002. However, the mean values of shoot length did not differ significantly at seedling stage in response to PEG induced osmotic stress (Table 1).

Drought stress of various durations at booting stage significantly influenced proline contents. A significant increase in proline contents was observed in leaf tissues of both R_1 somaclones and explant donor parent cv. GA-2002 in response to increasing duration of osmotic stress (Figure 1). Significantly higher proline contents were witnessed in the leaf samples of R_1 somaclones collected on the 4th, 6th or 8th day of water stress with corresponding proline contents of 4.02, 6.3 and 7.52 µmolg⁻¹ fr.wt., respectively. Nevertheless, non-significant differences were recorded for proline contents under control conditions and for stress duration of 2 days. Similarly, an increasing trend was observed for protein accumulation with escalated duration of water stress up to 6 days. However, the protein contents of the leaf samples of both R_1 somaclones and their explant donor parent cv. GA-2002 collected on the 8th day of stress were declined. The R_1 somaclones tend to accumulate the highest protein content (4.36 µmolg⁻¹ dry wt.) in response to water stress of 6 days, which were significantly higher than the parents at the same level of water stress (3.58 µmolg⁻¹ dry wt.; Figure 2).

The sugar and K^+ contents tend to increase with increasing severity of water stress. Stress-induced increase in sugar contents by the R_1 somaclones and

parent cv. GA-2002 was more comparable on the 4th, 6th and 8th day of withholding water (Figure 3), showing significantly more sugar accumulation by the R_1 somaclones than the explant donor parent. Similarly, the K^+ contents of both R_1 somaclones and their parent cv. GA-2002 were increased with increasing duration of water stress (Figure 4). The K^+ contents of R_1 somaclones (14.77 mgg^{-1} dry wt.) and their parent (15.33 mgg^{-1} dry wt.) were statistically at par under control conditions and varied significantly in response to 2nd, 4th, 6th and 8th day of water stress being higher in R_1 somaclones (Figure 4).

Table 1: Response of R_1 somaclones and explant donor parent cv. GA-2002 to PEG-6000 induced osmotic stress at seedling stage

	Control	PEG-6000	Mean
Root length (cm)			
Parent cv. GA-2002	14.32a	8.31c	11.32b
R_1 somaclones	14.36a	10.67b	12.52a
Mean	14.34a	9.50b	
Shoot length (cm)			
Parent cv. GA-2002	16.77a	12.94c	14.86a
R_1 somaclones	16.31a	14.62b	15.47a
Mean	16.54a	13.78b	
Root: Shoot			
Parent cv. GA-2002	0.854	0.643	0.748b
R_1 somaclones	0.882	0.730	0.806a
Mean	0.868a	0.686b	
RWC			
Parent cv. GA-2002	86.42a	76.60c	81.51b
R_1 somaclones	85.99a	82.29b	84.14a
Mean	86.21a	79.44b	

LSD Values			
	Plant material (P)	Stress (S)	P × S
Root	0.5662	0.5662	0.8007
Shoot	0.7223 [NS]	0.7223	1.0215
Root:Shoot	0.0535	0.0535	0.0757 [NS]
RWC	2.5102	2.5102	3.5499

[NS] Non-significant

Entries sharing similar letters do not differ significantly at 5 % probability level

The osmotic stress significantly affected leaf chlorophyll contents and a progressive decline in chlorophyll dynamics was observed with rising episode of water
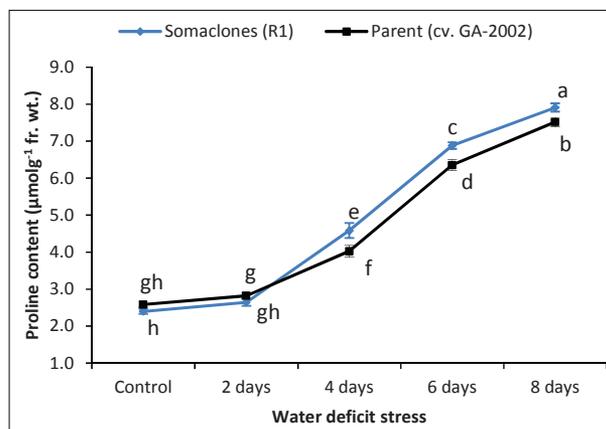

Figure 1: Proline contents (μmolg^{-1} fr. wt.) of R_1 somaclones and their parent wheat cv. GA-2002 in response to various durations of water stress at booting stage. Vertical bars represent standard errors. Bars sharing similar letters do not differ significantly (p < 0.05)
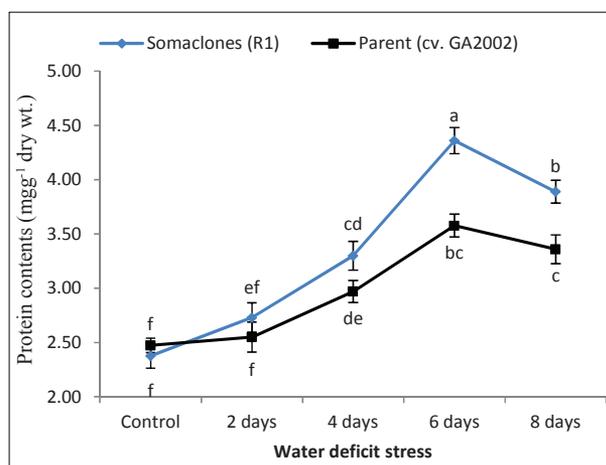

Figure 2: Protein contents (mgg^{-1} dry wt.) of R_1 somaclones and their parent wheat cv. GA-2002 in response to various durations of water stress at booting stage. Vertical bars represent standard errors. Bars sharing similar letters do not differ significantly (p < 0.05)

stress (Table 2). The maximum chlorophyll content (mean) was recorded in control plants (2.89 mgg^{-1} fr. wt.) whereas the minimum (1.30 mgg^{-1} fr. wt.) was on the 8th day of water stress. However, non-significant differences were recorded for mean chlorophyll contents of R_1 somaclones and parent cv. GA-2002 subject to osmotic stress (Table 2). Similarly, a declining trend was also observed for MSI in response to escalated duration of water stress showing mean maximum MSI under control

conditions and the least on 8th day of water stress. The mean MSI (74.51 %) of R_1 somaclones was significantly

higher than that of parent cv. GA-2002 (71.07 %) in response to simulated water stress (Table 2).

Table 2:   Comparative physio-chemical responses of *in vitro* selected R_1 somaclones and their parent cv. GA-2002 in response to simulated water stress of various durations at booting stage

	Control	2 days	4 days	6 days	8 days	Mean
			Water stress in days			
Chlorophyll contents (mgg⁻¹ fr. wt.)						
Parent cv. GA-2002	2.94	2.83	2.41	1.85	1.25	2.26
R_1 somaclones	2.84	2.81	2.49	1.98	1.35	2.30
Mean	2.89a	2.82a	2.45b	1.92c	1.30d	
Leaf membrane stability index (MSI)						
Parent cv. GA-2002	85.11	81.57	74.32	64.68	49.65	71.07b
R_1 somaclones	86.31	83.56	77.91	70.56	54.19	74.51a
Mean	85.71a	82.57a	76.11b	67.62c	51.92d	
LSD values						
	Plant material (P)		Stress (S)	P × S		
Chlorophyll contents	0.057 NS		0.090*	0.128 NS		
MSI	2.011*		3.180*	4.496 NS		

NS Non-significant; * significant

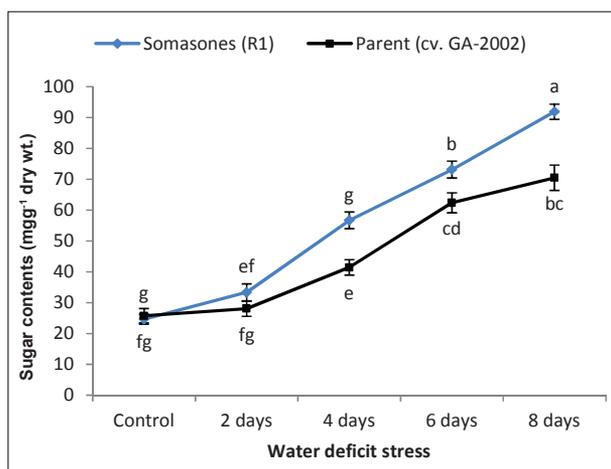Entries sharing similar letters do not differ significantly at 5 % probability level

Figure 3:   Sugar contents (mgg⁻¹ dry wt.) of R_1 somaclones and their parent wheat cv. GA-2002 in response to various durations of water stress at booting stage. Vertical bars represent standard errors. Bars sharing similar letters do not differ significantly (p < 0.05)
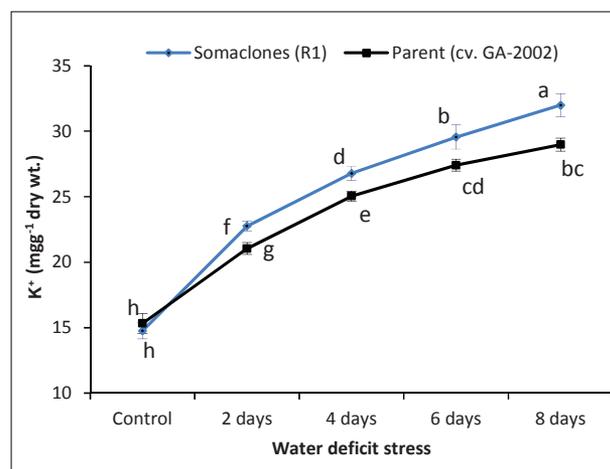

Figure 4:   K⁺ contents (mgg⁻¹ dry wt.) of R_1 somaclones and their parent wheat cv. GA-2002 in response to various durations of water stress at booting stage. Vertical bars represent standard errors. Bars sharing similar letters do not differ significantly (p < 0.05)

Drought has significant effects on the physiology of cereal crops. The plants demonstrate various morphological, physiological, biochemical and molecular responses to tackle drought stress. At morphological level, shoot and root growth, its density, proliferation and size are most affected and are the key components of plant adaptation to drought (Farooq *et al*., 2009). Retarded shoot growth and increased root growth are the morphological adaptive traits in plants under water stress conditions (Dhanda *et al*., 2004). The root and shoot length of wheat plant decrease in response to moisture deficit stress, while root to shoot length ratio increase compared with that of well watered plants (Safarnejad, 2004). In wheat, up to 40 % increase in root to shoot length ratio has been reported in stressed plants compared to control. The increase in root to shoot dry matter/length ratio under water deficit conditions is not due to the increase in root mass but rather due to reduced shoot mass (Dhanda *et al*., 2004). The results of the present study suggested that root and shoot growth was suppressed more in explant donor parent cv. GA-2002 in response to simulated water stress showing that they are more susceptible to drought. The findings are in accordance with earlier reports suggesting that somaclones regenerated from PEG tolerant calli are more drought tolerant due to better root and shoot growth under osmotic stress conditions (Safarnejad, 2004; Verma *et al*., 2013).

Under water deficit conditions at the metabolite level, amino acids, most notably proline increase to provide osmoprotective functions, prevent dissociation of enzymes and safeguard the cell from reactive oxygen species (ROS) (Kumar *et al*., 2011; Mehmood-ul-Hassan *et al*., 2013). Accordingly, genotypes which accumulate more proline are less prone to adverse effects of drought stress (Chandrasekar *et al*., 2000; Qayyum *et al*., 2017). The selected somaclones are also reported to accumulate more proline than parents with improved stress tolerance (Safarnejad, 2004; Verma *et al*., 2013).

Proteins and lipids are the major constituents of membranes. Drought induced metabolic changes associated with protein turnover have shown that rubisco, rubisco binding protein (RBP), rubisco activase (RA), dehydrins (DHN), ATP dependent calpain protease (Clp) proteins and some heat shock proteins (HSP) are substantially increased in both drought tolerant and susceptible cultivars of wheat in response to drought stress, with higher increment in tolerant ones (Demirevska *et al*., 2008; Qayyum *et al*., 2017). Therefore, under drought conditions, the protein contents are increased substantially in the root and shoot tissues of wheat (Tayeb & Ahmed, 2010) affirming the results

of the present study (Figure 2). The tendency of the R_1 somaclones to accumulate more protein content than the parent cv. GA-2002 (Figure 2) depicted their better drought tolerance.

Other than proline and protein, sugars also play a pivotal role in osmotic adjustment without inhibiting activity of the enzymes and ensure survival of plants under water deficit conditions. In addition, sugars help to stabilise the membrane system and keep proteins intact and functional under drought conditions (Sinay & Karuwal, 2014) and are thereby considered a reliable marker for assessing the level of drought tolerance in wheat (Cheng *et al*., 2015). The results showed an increasing trend for sugar accumulation and the response was more pronounced against moisture stress of 4th, 6th and 8th days, with significantly higher sugar contents portrayed by R_1 somaclones (Figure 3). A significant increment in the soluble sugar content of drought tolerant and sensitive genotypes has been reported in response to drought stress (Marcińska *et al*., 2013; Cheng *et al*., 2015; Qayyum *et al*., 2017). However, drought tolerant genotypes/plants tend to accumulate more soluble sugar contents than sensitive ones (Marcińska *et al*., 2013; Qayyum *et al*., 2017).

In addition, traits expected to improve adaptation of wheat to the Mediterranean environment also included accumulation of potassium in the leaves (Kusvuran, 2012). Potassium plays a pivotal role in economical yield, growth, protein biosynthesis, enzyme activation, photosynthesis, transpiration, stomatal regulation and osmotic adjustment (Farooq & Azam, 2001; Farooq *et al*., 2009). It is anticipated that about 60 % of osmotic adjustment in both mature and expanding leaves of wheat is accomplished by potassium salts (Kusvuran, 2012). Wheat genotypes differ in their ability to accumulate K^+ and the genotypes which tend to accumulate relatively more K^+ under water deficit conditions tend to endure drought stress in a better way (Farooq & Azam, 2001; Zhu *et al*., 2005). Undifferentiated cell/callus lines of drought tolerant genotypes of wheat are also reported to accumulate more intracellular K^+ than sensitive ones (Trivedi *et al*., 1991). Significantly higher accumulation of K^+ by the R_1 somaclones than the parent cv. GA-2002 with mounting duration of water stress (Figure 4) showed that they are more drought tolerant, and it is supported by the findings of Begum and Islam (2015).

The chlorophyll content provides insight regarding photosynthesis particularly under water deficit conditions. Over production of ROS is triggered by excessive energy absorption by the photosynthetic apparatus and is avoided

by the plants through degradation of light absorbing pigments accompanied by increased reflectance of the incident radiation by changing the green colour of the leaf into yellow (Kumar *et al.*, 2011) and is considered an important drought adaptive trait. Hence, water stress results in pigment degradation and leads to irreversible damage to the photosynthetic machinery/chlorophyll (Bogale *et al.*, 2011). Therefore, chlorophyll contents tend to decline with increasing severity of drought stress (Chandrasekar *et al.*, 2000; Kumar *et al.*, 2011). The chlorophyll contents of R_1 somaclones and parent cv. GA-2002 declined with increasing duration of water stress (Table 2) and is in conformity with previous reports (Chandrasekar *et al.*, 2000; Kumar *et al.*, 2011; Razzaq *et al.*, 2013a; 2013b). Under drought conditions, the drought sensitive plants lose chlorophyll and senescenced earlier than drought tolerant ones (Saeedipour & Moradi, 2011). Accordingly, the genotypes which maintain higher chlorophyll contents under limited supply of moisture are considered more drought tolerant (Chandrasekar *et al.*, 2000; Saeedipour & Moradi, 2011). The regenerated somaclones (R_1) did not show improved chlorophyll stability than their explant donor parent under water stress conditions (Table 2), since the somaclones are not always superior to explant donor parents for most of the agronomic and quality parameters (Hanson *et al.*, 1994). Contrary to the above, some reports have shown variations in chlorophyll contents of regenerated clones of wheat (Yasmin *et al.*, 2009). For example, Hsissou and Bouharmont (1994) regenerated drought tolerant plants of durum wheat with improved chlorophyll fluorescence, mainly because of differences of protocol and plant material used. It is revealed that R_1 somaclones may cope with water stress by different adaptive strategies other than chlorophyll stability index; given that, drought tolerance is a complex trait and all drought adopted traits are not always expressed simultaneously in every drought tolerant genotype (Chandrasekar *et al.*, 2000).

Soil moisture deficit also results in dehydration of the cells accompanied by electrolyte leakage due to strain exerted by increased intercellular concentration of solutes on membranes and macromolecules. The membrane stability index (MSI) is a measure of the intactness of the membranes' structure against electrolyte leakage under drought stress conditions, and the index of membrane injury is proportional to the post desiccation electrolyte leakage from the membrane systems (Ali *et al.*, 2009). Proteins and lipids are the major constituents of membranes. Reactive oxygen species (ROS), predominantly hydrogen peroxide (H_2O_2) and superoxide radicals are increased under soil moisture deficit conditions and cause oxidative damage to proteins, nucleic acids and lipids (Al-Ghamdi, 2009), thereby interrupt association between the lipid and protein bilayer. Therefore, membrane systems are the major loci prone to desiccation injury, and the transport ability of the membranes is impaired. Many reports suggested that the MSI declines with increasing moisture deficit stress (Chandrasekar *et al.*, 2000; Razzaq *et al.*, 2013a; 2013b). However, drought tolerant genotypes are shown to maintain a higher MSI than drought susceptible ones (Chandrasekar *et al.*, 2000). The ability of the R_1 somaclones to maintain higher MSI than the parent cv. GA-2002 (Table 2) showed that they are more drought tolerant. Hsissou and Bouharmont (1994) have also regenerated drought tolerant somaclones of wheat from osmotic stress tolerant callus lines with less electrolyte leakage (high membrane stability) than parental lines.

Based on the morpho-physiological responses of R_1 somaclones and explant donor parent cv. GA-2002 under drought conditions, it is evident that R_1 somaclones regenerated from PEG-6000 tolerant callus lines have improved drought tolerance than the parent line. It is likely that the surviving tolerant calli may acclimate to osmotic stress when exposed to two consecutive passages and the cellular components of stress tolerance were inherited to R_1 somaclones thereafter (Lutts *et al.*, 2001). The improved drought tolerance of R_1 somaclones is also in accordance with the findings of many other researchers. For example, drought tolerant somaclones (R_1 and R_2) of sugarcane were developed by Taghian (2002) using PEG-6000 or NaCl as stressing agents. Mahmood *et al.* (2012a; 2014) and Verma *et al.* (2013) screened PEG-6000 osmotic stress tolerant calli of wheat and rice, respectively, and regenerated drought tolerant plants thereafter from selected callus lines. Similarly, drought tolerant somaclones of maize were regenerated from PEG-6000 tolerant calli by El-Aref (2002) and Matheka *et al.* (2008).

CONCLUSION

The ability of the *in vitro* selected R_1 somaclones to exhibit more root length, shoot length, root to shoot length ratio and RWC under simulated drought stress at seedling stage, and to accumulate relatively higher osmolites, K^+ contents and to maintain relatively higher MSI in response to drought stress at booting stage than the explant donor parent cv. GA-2002 indicated their improved drought tolerance. The study signifies that immature embryo culture in the presence of a suitable concentration of osmoticum can be employed to exploit somaclonal variation for improving drought tolerance

of wheat. It is suggested that to confirm whether the acquired drought tolerance of R_1 somaclones is of genetic or epigenetic nature, the stability of drought tolerance based on morpho-physiological drought adapted traits and yield parameters should be tested in R_2 and R_3 generations.

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